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TITLE: Identifying Epigenetic Modulators of Resistance to ERK Signaling Inhibitors

PRINCIPAL INVESTIGATOR: Emily Bernstein, PhD

CONTRACTING ORGANIZATION: Icahn School of Medicine at Mount Sinai
New York, NY 10029

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Emily Bernstein, PhD

email: emily.bernstein@mssm.edu

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Icahn School of Medicine at Mount Sinai

One Gustave L. Levy Place
New York, NY 10029

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Recent studies uncovered a role for chromatin regulators in response to targeted therapies in cancer. However, a global and unbiased approach to decipher the epigenetic mechanisms underlying melanoma drug resistance has yet to be reported. Our studies are revealing an 'epigenomic map' of drug resistant cells and will allow us to uncover key epigenetic regulators underlying ERK signaling inhibitor resistance of malignant melanoma. Since our current knowledge of the chromatin biology underlying the process of drug resistance to ERK signaling inhibitors is essentially nil, we are taking innovative high-throughput approaches to study this intriguing and critical issue. Our work over the past year has identified chromatin mediators that when depleted form cells, enhance the melanoma drug resistance phenotype. These are novel findings to our knowledge and implicate distinct mechanisms of chromatin regulation in this process. This approach is also highly clinically relevant, as it will provide rationale for combining therapy targeted against identified epigenetic modulators with current therapies.

15. SUBJECT TERMS

None Listed

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Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Because of its resistance to all existing treatments, metastatic melanoma remains one of the most lethal forms of cancer. However, melanoma treatments have recently advanced with the application of new drugs that inhibit key biological targets, such as ERK signaling inhibitors. A large proportion of patients respond remarkably to these treatments, but relapse within a 6-9 month period because tumors inevitably acquire resistance. We hypothesize that genome-wide epigenetic alterations lead to inappropriate gene expression programs and altered chromatin structure, resulting in melanoma drug resistance. Here, we propose to uncover the epigenetic mechanisms that play a significant role in melanoma cells that have acquired resistance to ERK signaling inhibitors. By mapping their epigenomic landscape we will generate global insight into the epigenetic changes in melanoma cells that have acquired drug resistance. Moreover, we anticipate that our shRNA screen targeting chromatin-related factors in human melanoma cells undergoing resistance will uncover novel and critical therapeutic epigenetic targets.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Melanoma, MAPK signaling, drug resistance, epigenome, shRNA screen, chromatin

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the USAMRAA Grants Officer whenever there are significant changes in the project or its direction.

- What were the major goals and objectives of the project?
- What was accomplished under these goals?
- What opportunities for training and professional development did the project provide?
- How were the results disseminated to communities of interest?
- What do you plan to do during the next reporting period to accomplish the goals and objectives?

What were the major goals of the project?

<u>AIM 1. Map the epigenomic landscape of melanoma cells that have acquired resistance to ERK signaling inhibitors. (Months: 1-16)</u>

Milestone 1. Gain insight into the epigenetic changes associated with drug resistance in melanoma cells. ONGOING

Milestone 2. Identify gene expression programs and 'driver' genes in resistant cells based on promoter and enhancer chromatin profiles. ONGOING

<u>AIM 2: Identify novel epigenetic regulators that mediate ERK signaling inhibitor resistance via high throughput loss-of-function assays. (Months: 8-24)</u>

Milestone 3. Establish a human 'Chromatinome' shRNA library to screen for epigenetic drivers of melanoma resistance. COMPLETED 08/2014

Milestone 4. Perform shRNA screen for epigenetic regulators in melanoma resistance. COMPLETED 05/2015

Milestone 5. Identify potential epigenetic drivers of melanoma resistance for further investigation. COMPLETED 06/2015

Milestone 6. Hits from the shRNA screen will be validated for their roles in regulating drug resistance. ONGOING

Milestone 7. Potential epigenetic drug targets will be identified. ONGOING

Milestone 8. A manuscript describing our findings will be submitted to a high-impact journal. WITHIN THE NEXT 12 MONTHS

What was accomplished under these goals?

AIM 1. Map the epigenomic landscape of melanoma cells that have acquired resistance to ERK signaling inhibitors. (Months: 1-16)

In order to pursue epigenetic changes of melanoma drug resistance, we have generated drug resistant cells (clones) in multiple BRAF^{V600E} mutant melanoma cell lines, including SKMEL239, 501Mel and A375 using clinically relevant compounds (Dabrafenib (RAFi), Dabrafenib+Trametinib (RAFi+MEKi combination)). These cells contain the BRAF^{V600E} mutation and were exposed to high doses of RAFi (0.5, 1 or 2μM) or the RAFi+MEKi combination, which inhibits ERK signaling and induces cell cycle arrest and death (**Figure 1**). However, after 4 to 6 weeks of continuous exposure to Dabrafenib or the combination, several resistant populations arose. Intriguingly, we have noticed striking variation in the degree of pigmentation of these clones, suggesting that gene expression programs are indeed altered in resistant cells, possibly mediated by chromatin changes. Moreover, by profiling histones post-translational modifications (PTMs) in several resistant clones, we identified changes in histone acetylation and methylation in these cells compared to the parental and sensitive cell line, suggesting significant epigenetic changes in drug resistant cells (**Figure 2**).

Because of these identified changes, we have focused our efforts on probing the epigenomic landscape of resistance in RAFi resistant cells (compared to the parental cell line) by ChIP-seq analysis. ChIP-seq is a high-throughput technique used successfully in our laboratory to map histone PTMs and histone variants throughout the genome, which has provided great insight into cell-type specific gene regulation. Such experiments will allow us to decipher how the chromatin state of resistant cells regulates gene expression and/or non-coding regions of the genome, and will provide us with key gene targets that may confer resistance. To date, we have performed ChIP for the following histone PTMs in SKMEL239 (parental) and one resistant cell line (Clone 1 in **Figure 2B**): H3K4me1, H3K4me3 and H3K27ac (markers of enhancer and promoter elements) as well as H3K9ac and H3K9me3 (functionally opposing histone modifications we find to be altered in resistant clones, **Figure 2**). Sequencing has been performed for H3K4me1, H3K4me3 and H3K27ac libraries to date and the data is currently being analyzed. This process takes approximately one month's time, and we have established an excellent working pipeline for these analyses. We anticipate differences in promoter activation and enhancer usage between the sensitive and resistant lines and eagerly await these results. The epigenomic 'landscape' of resistance to MAPK inhibitors will generate a global insight into the epigenetic changes in melanoma cells that have acquired drug resistance.

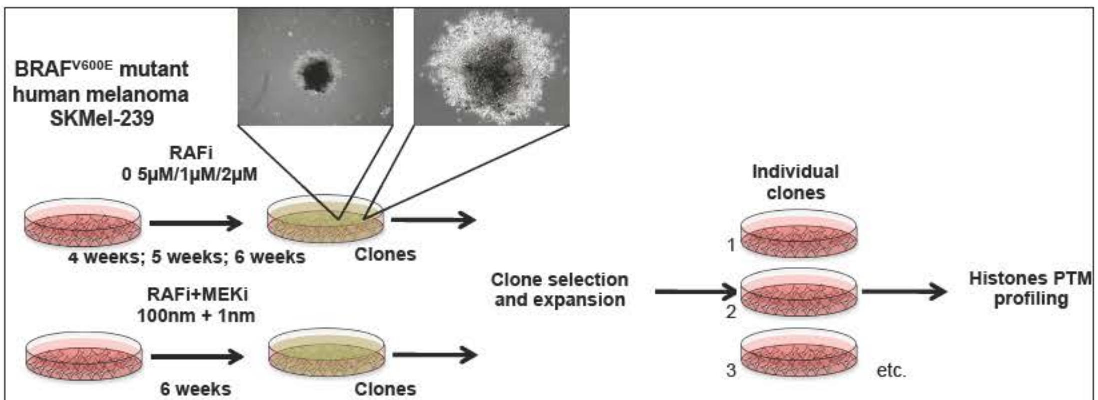

Figure 1: Generation of melanoma drug resistant cells. SKMel-239 (BRAF^{V600E}) melanoma cells treated with clinically relevant compounds Dabrafen b (RAFi) alone or the Dabrafen b+Trametin b (RAFi+MEKi combination) to generate resistant clones. After, several weeks of treatment with the indicated concentrations, individual clones were expanded and histone PTM profiling was performed (Figure 2).

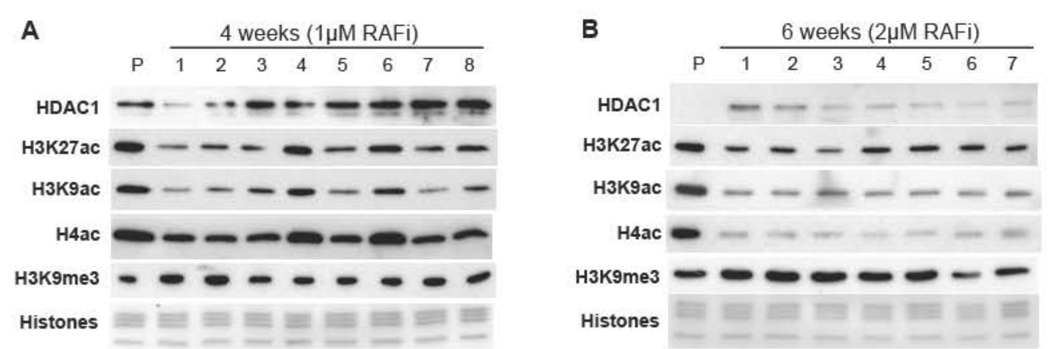

Figure 2: Histone acetylation changes in RAFi-resistant melanoma cells. SKMel-239 RAFi resistant clones selected after four weeks (Panel A, clones 1-8) or six weeks (Panel B, clones 1-7) of treatment with the indicated concentrations. Clones were compared to the sensitive parental line (P). Immunoblot for HDAC1 reveals higher levels in RAFi resistant clones compared to sensitive parental cells. Immunoblot for the histone PTMs H3K27, K9 and H4 acetylation shows a decrease in all resistant clones compared to the parental cells while H3K9me3 shows an increase. Total histones used as a loading control.

We hypothesis that alterations of the chromatin state can be involved in mechanisms underlying drug resistance

in melanoma cells Our preliminary data allow us to propose a model whereby epigenetic modifications in acquired melanoma drug resistance may trigger a more aggressive state. Therefore, identifying chromatin factors through unbiased approaches may lead to novel targets for therapy of melanoma patients and will reveal a potential for the use of epigenetic inhibitors as targets that can also be used in combination with ERK signaling inhibitors (Aim 2).

<u>AIM 2: Identify novel epigenetic regulators that mediate ERK signaling inhibitor resistance via high throughput loss-of-function assays. (Months: 8-24)</u>

We performed a loss-of-function screen targeting chromatin-related factors in BRAF^{V600E} human melanoma cells. Because we have generated drug resistance clones for Aim 1, we have extensive experience in the system used for performing the screen (i.e. **Figure 1**), with some alterations such as infections of the libraries and sorting of infected cells. Cells were transduced with the 'Chromatinome' library as a pool (**Figure 3**). A low multiplicity of infection (MOI) of vector was used to ensure that each cell is only transduced with a single vector. All library vectors contain a GFP marker in order to quantitate viral titers via FACS. GFP positive cells were sorted by flow cytometry, cultured for a week for loss-of-function to take effect and then split into 10 cm plates. A total of 30 plates were seeded for library-infected SKMEL239 cells: 10 plates were subsequently treated with RAFi, 10 plates with RAFi/MEKi, and 10 plates with DMSO-treated as a control (**Figure 3**). Non-transduced cells were grown in parallel treated with RAFi or RAFi/MEKi as control for the entire process, including the timing of appearance of resistance colonies. See **Figure 3** for the flow of the screening approach.

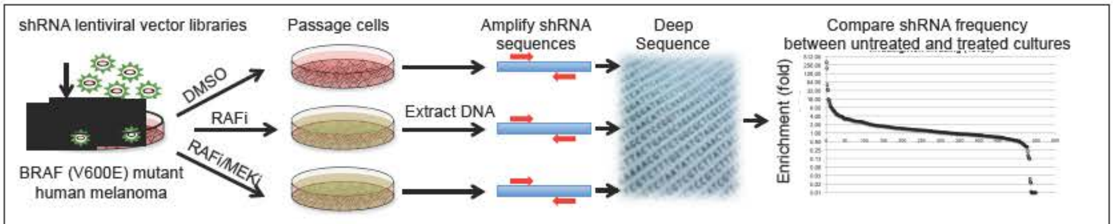

Figure 3. Experimental flow of pooled parallel loss-of-function assay for epigenetic modulators of ERK signaling inhibitor resistance.

To date, we have performed the screen as two biological replicates, which revealed novel and critical epigenetic regulators of resistance to RAFi and RAFi+MEKi (**Figure 4**). Our screen has identified chromatin factors that play a role in promoting drug resistance including SIRT6, an NAD-dependent histone deacetylase with known roles in metabolism and DNA repair, NSD1 and SETDB1 (histone methyltransferases for H3K36me3 and H3K9me3, respectively), as well as ubiquitin regulators such as RNF2 and USP22 (E3 ligase and deubiquinase, respectively). Other hits include genes involved in MAPK signaling, which serve as controls for the screens. We are now utilizing individual assays to validate the results of our pooled screen. The cells will be cultured in the presence of RAFi, RAFi+MEKi and the number and timing of appearance of resistant cells will be scored and compared to the control. In addition, by using human melanoma short-term cultures and patient samples pre- and post-treatment, top hits will be validated by Immunohistochemistry (IHC) staining and immunoblot. We are very excited about these discoveries and their potential for understanding the biology of melanoma drug resistance.

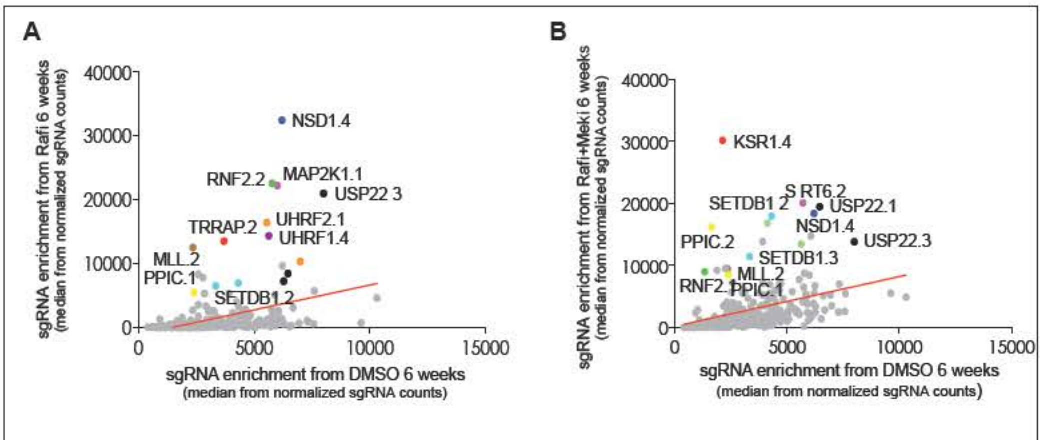

Figure 4: Loss of function screen in SKMel-239 melanoma cells reveals chromatin factors whose loss promotes resistance to MAPK signaling inhibitors. A. Scatterplot showing enrichment of specific sgRNAs after 6 weeks of Dabrafenib treatment. **B.** Scatterplot showing enrichment of specific sgRNAs after 6 weeks of Dabrafenib+Trametinib treatment. Genes indicated represent significant hits from the screen that are currently being validated.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Over the next reporting period, as per Aim 1 we will continue our ChIP-seq efforts and data analysis to decipher the epigenomic landscape of melanoma drug resistance. We will also extend our ChIP-seq experiments to additional drug resistant clones to account to for heterogeneity in epigenomes of drug resistant cells. As per Aim 2, we will validate the candidates identified in the pooled loss-of-function screen. We also have unexploited libraries that can be used to screen for additional factors that play a role in drug resistance, which are focused on transcription factors and transcriptional regulators. While, beyond the scope of the proposed studies, we also aim to functionally dissect the roles of top validated hits from the screen.

4. IMPACT: This component is used to describe ways in which the work, findings, and specific products of the project have had an impact during this reporting period. Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- the development of the principal discipline(s) of the project;
- other disciplines;
- technology transfer; or
- society beyond science and technology.

What was the impact on the development of the principal discipline(s) of the project?

In the last 12 months, we have made significant progress in addressing the goals of our proposal. The proposed aims were designed to understand the epigenetic mechanisms underlying melanoma drug resistance. Recent advances in targeted therapies, such as BRAF and MEK inhibitors, have raised hope for a melanoma cure. However, while a large proportion of patients respond remarkably to these treatments, tumors inevitably acquire resistance and patients relapse within a 6-9 month period. This continues to be a major clinical challenge. Our work suggests that epigenetic factors indeed play a role in melanoma drug resistance, and provide new targets for therapy. Because we are taking unbiased approaches to reveal the mechanisms of melanoma drug resistance, we are likely to uncover new and exciting avenues for drug discovery.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information on participants:

- what individuals have worked on the project?
- has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?
- what other organizations have been involved as partners?

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

Name:	Emily Bernstein
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	EBERNSTEIN
Nearest person month worked:	2
Contribution to Project:	Dr. Bernstein provided oversight and guidance of the project. She met with postdoctoral fellows on a weekly basis.
Funding Support:	N/A

Name:	Flavia Ghiraldini
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	FGHIRALDINI
Nearest person month worked:	3
Contribution to Project:	Dr. Ghiraldini has played an integral role in identifying and validating hits from the screens (Aim 2).

Funding Support:	N/A

Name:	Chiara Vardabasso
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	VARDABASSO
Nearest person month worked:	7
Contribution to Project:	Dr. Vardabasso has been investigating the histone changes in melanoma progression and drug resistance. She is also involved in ChIP-seq efforts (Aim 1).
Funding Support:	N/A

Name:	Dan Hasson
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	HASSON
Nearest person month worked:	5
Contribution to Project:	Dr. Hasson has been leading the ChIP-seq efforts to map the epigenome of sensitive vs. drug resistant cells (Aim 1).
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Emily Bernstein:

Melanoma Research Alliance 0.36 calendar 05/01/2015-04/30/2018

Harnessing the epigenome for melanoma oncogene discovery

The proposed study aims to characterize enhancer-associated genes in melanoma cells with biological significance via functional targeted loss-of function screens.

Role: PI

Harry J. Lloyd Charitable Trust 0.36 calendar 05/01/2015-04/30/2017

Melanoma oncogene discovery via an epigenetic approach

We propose to generate unprecedented maps of histone modifications, chromatin regulators, and the transcriptome of human melanocytes and melanoma relevant to enhancer function.

Role: PI

OVERLAP: NONE

Brian Brown:

R01AI113221-01A1 (Brown) 1.56 cal. months 03/01/2015 - 02/28/2020

NIH/NIAID

Modulating Immunity to Nucleic Acids and Inducing Tolerance by Gene Transfer.

The Specific Aims of this project are to: (1) Identify the function of miR-126 in the innate response to gene therapy, (2) Establish the role of VEGFR2 signaling in the innate response to gene delivery, and (3) Evaluate pDCs as a target for a tolerogenic gene-based vaccine.

Role: PI

BHAP (Brown) 1.8 cal months 07/01/2014 – 05/31/2016

Bayer Hemophilia Award Program

Targeted correction of hemophilia A using CRISPR-mediated editing

The Specific Aims of the project are to: (1) Insert a human FVIII cDNA into the Rosa26 locus of the mouse genome using the CRISPR-Cas9 system, and (2) Insert a human FVIII cDNA into the AAVS1 locus of the human genome using the CRISPR-Cas9 system.

Role: PI

RGP009/2014 (Brown) 1.8 cal months 06/01/2014 – 05/31/2017

Human Frontier Science Program (HFSP)

Deciphering non-coding RNA regulatory networks and their role in cancer cell biology

The Specific Aims of the project are to: (1) Identify non-coding RNAs with sponge activity, (2) Determine the co-factors that affect non-coding RNA regulation of microRNAs, and (3) Determine the relevance of linear and circular non-coding RNAs to cancer cell biology.

Role: PI

OVERLAP: NONE

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: None